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Evolution of Developmental Control Mechanisms

Initiation and patterning of the snake dentition are dependent on Sonic Hedgehog signaling

Marcela Buchtová [a,1], Gregory R. Handrigan [a], Abigail S. Tucker [c], Scott Lozanoff [d], Liam Town [b], Katherine Fu [a], Virginia M. Diewert [a], Carol Wicking [b], Joy M. Richman [a,*]

[a] Department of Oral Health Sciences, University of British Columbia, Vancouver, BC, Canada
[b] Institute for Molecular Bioscience, The University of Queensland, Brisbane, Australia
[c] Department of Craniofacial Development and Orthodontics, King's College London, London, UK
[d] Department of Anatomy, University of Hawaii, USA

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ABSTRACT

Here we take the first look at cellular dynamics and molecular signaling in the developing snake dentition. We found that tooth formation differs from rodents in several respects. The majority of snake teeth bud off of a deep, ribbon-like dental lamina rather than as separate tooth germs. Prior to and after dental lamina ingrowth, we observe asymmetries in cell proliferation and extracellular matrix distribution suggesting that localized signaling by a secreted protein is involved. We cloned *Sonic hedgehog* from the African rock python *Python sebae* and traced its expression in the species as well as in two other snakes, the closely-related *Python regius* and the more derived corn snake *Elaphe guttata* (Colubridae). We found that expression of *Shh* is first confined to the odontogenic band and defines the position of the future dental lamina. *Shh* transcripts in pythons are progressively restricted to the oral epithelium on one side of the dental lamina and remain in this position throughout the prehatching period. *Shh* is expressed in the inner enamel epithelium and the stellate reticulum of the tooth anlagen, but is absent from the outer enamel epithelium and its derivative, the successional lamina. This suggests that signals other than Shh are responsible for replacement tooth formation. Functional studies using cyclopamine to block Hh signaling during odontogenesis prevented initiation and extension of the dental lamina into the mesenchyme, and also affected the directionality of this process. Further, blocking Hh signaling led to disruptions of the inner enamel epithelium. To explore the role *of Shh* in lamina extension, we looked at its expression in the premaxillary teeth, which form closer to the oral surface than elsewhere in the mouth. Oral ectodermal *Shh* expression in premaxillary teeth is lost soon after the teeth form reinforcing the idea that *Shh* is controlling the depth of the dental lamina. In summary, we have found diverse roles for *Shh* in patterning the snake dentition but, have excluded the participation of this signal in replacement tooth formation.

Introduction

The genetic basis for forming replacement teeth in amniotes is poorly understood, in part because we do not have convenient animal models in which to study this process. Conventional mammalian models such as mice and rats only form a single generation of teeth. The multi-generational reptilian dental lamina appears to share some characteristics with some mammalian dentitions that have 2 generations (e.g., primates and Soricidae) in that replacement teeth appear to bud from the outer layers of previous generations (Buchtová et al., 2007). Furthermore, reptiles are more closely related to mammals than are fish, the other main model in which to study tooth replacement (Fraser et al., 2006a,b, 2004; Huysseune, 2006; Huysseune and Thesleff, 2004). Therefore developing reptilian models of tooth development will help to address the knowledge gaps in successional tooth formation.

The sequence of reptilian tooth replacement has been described in crocodilians (Bolk, 1912; Röse, 1892; Woederman, 1919, 1921), tuatara (Westergaard and Ferguson, 1986; Westergaard and Ferguson, 1987, 1990) and lizards (Delgado et al., 2005; Osborn, 1971). More recent reptilian tooth studies have begun to characterize interspecific differences in enamel (Delgado et al., 2006; Diekwisch et al., 2002; Satchell et al., 2002; Shintani et al., 2002, 2006; Sire et al., 2007; Wang et al., 2006) and cementum composition (Luan et al., 2006). No one, however, has studied the molecular specification of tooth pattern in a reptile. Thus we know little about the evolutionary conservation or divergence of key morphogenetic signals in reptiles.

* Corresponding author. Life Sciences Institute, University of British Columbia, 2350 Health Sciences Mall, Vancouver, B.C., Canada V6T 1Z3.
E-mail address: Richman@interchange.ubc.ca (J.M. Richman).
[1] Present address: Institute of Animal Physiology and Genetics, v.v.i. Academy of Sciences of the Czech Republic, Brno, Czech Republic; Department of Anatomy, Histology and Embryology, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic.

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In choosing a reptilian model system, we looked for a dentate and oviparous species (for easier access to embryos). During the course of evolution teeth were retained in the majority of mammals (with the sole exception of anteaters). However, the dental status of reptiles became much more varied. Turtles and birds completely lost their dentition, whereas tuatara, squamates (lizards and snakes) and crocodilians have not only retained teeth, but in some cases have teeth in the roof of the mouth in addition to those at the margins. Within the squamates, palatine teeth are most well-developed in snakes. Several lizard species have pterygoid and palatine teeth, but these are generally quite small (Mahler and Kearney, 2006). By comparing the development of the palatine teeth to that of marginal teeth in the snake, we test the hypothesis that similar developmental cues are replicated between tooth rows on the upper jaw.

Furthermore, the teeth of the snake are uniformly unicuspid which should reduce the likelihood that there are differences in expression of patterning genes in different regions of the mouth. In choosing a type of snake model, we excluded the fanged, venomous snakes since they have highly specialized tooth morphology. The results obtained from studying a non-venomous snake can be more easily generalized to other reptiles and vertebrates at large.

Teeth initiate as a result of epithelial–mesenchymal interactions with the epithelium providing the initial instructive signal (Lumsden, 1988; Mina and Kollar, 1987). Most of what we know about the molecular signaling during early stages of tooth development comes from excellent studies in rodents (Jernvall and Thesleff, 2000; Matalova et al., 2004; Thesleff and Mikkola, 2002). The epithelial signals include *Shh, Bmp4* and *Fgf8* (Thesleff, 2003). The expression of these genes along with *Pitx2* occurs at specific locations in the oral ectoderm and predicts sites of future tooth morphogenesis. The current model for mammalian tooth patterning is that there is first an odontogenic band that can only be recognized by differential gene expression and from this band, thickened epithelial placodes develop. The dental epithelial placodes initiate expression of transcription factors *Msx1* and *Pax9* in the underlying mesenchyme. In rodents, the dental lamina forms in discrete regions of the dental arch, giving rise to incisors in the anterior and molars in the posterior. In the interdental region, transitory tooth buds form and then regress (Peterkova et al., 2002) due to repression of signals such as FGFs (Klein et al., 2006). It is not clear whether the tooth forming epithelium of reptiles is discrete as in rodents or continuous along the jaw, nor which signals are required for setting up the specialized dental epithelium.

Once the mesenchyme condenses around the dental epithelium, the instructive signaling for tooth shape passes to the mesenchyme (Kollar and Baird, 1970). A multilayered enamel organ develops surrounding the mesenchymally-derived dental papilla. The innermost layer of the enamel organ, the inner enamel epithelium, forms the morphogenetic signaling centre known as the enamel knot (Jernvall et al., 1994). The knots are where folding of the molar tooth germ occurs leading to cusp formation (Jernvall and Thesleff, 2000). It is not known whether enamel knots are present during development of single-cusped canines or the pointed teeth of cetaceans. The enamel knot is characterized by absent cell proliferation (Casasco, 1996; Shigemura et al., 1999), high apoptosis (Shigemura et al., 1999; Vaahtokari et al., 1996) and restricted gene expression of signals such as *Shh* (Bitgood and McMahon, 1995). The molariform teeth develop secondary enamel knots during the bell stage and also at this stage the secretion of dentin begins. Once dentin forms, ameloblasts differentiate from the inner enamel epithelium and begin to secrete enamel. *Shh* is also expressed in ameloblasts and conditional inactivation of *Shh* in the ectoderm in mice leads to enamel dysplasia (Dassule et al., 2000). *Shh* is specifically upregulated in the persistent dental lamina of the *Runx2* knockout mice (Wang et al., 2005). This result suggests that in other mammals *Shh* may promote the formation of second generation teeth.

Humans have 20 teeth in which two generations are present (incisors, canines and bicuspids) and 12 teeth in which only one generation forms (the molars). There are several genetic disorders that affect tooth generations in humans, such as ectodermal dysplasia (hypodontia or oligodontia; Mikkola and Thesleff, 2003) and cleidocranial dysplasia (multiple supernumerary teeth; Jensen and Kreiborg, 1990; Mundlos et al., 1997; Otto et al., 1997). Interestingly, in many cases the primary dentition is intact, while the formation of the second generation of teeth is affected. These data suggest that the same signals that initiate primary teeth are not necessarily responsible for forming permanent successional teeth. One of the long-term goals of our research is to not only identify the signals involved in successional tooth formation but then to go on and to experimentally test the function of these proteins in reptilian models.

In this paper we chose to develop the snake as a model for studying dental development. One of the main reasons is that our previous work had shown that reiterative signaling can be studied not just once but up to 4 times in the prehatching snake embryo (Buchtová et al., 2007). As a first step, we describe the many unique aspects of tooth development in three species of non-venomous snakes, two from the same genus, *Python sebae* and *Python regius*, and one from the more derived Colubroid family, *Elaphe guttata*. We then cloned *Shh* from *P. sebae*, mapped its expression in the three snake species and tested gene function by blocking hedgehog signaling in dental explants. The data support roles for *Shh* in tooth initiation and determination of the distinct asymmetry of the reptilian dental laminae, but not in the induction of generational teeth.

Materials and methods

Snake egg incubation and embryo fixation

In this study we used three snake species, African rock python (*P. sebae*), ball python (*P. regius*) and the corn snake (*E. guttata*). *P. sebae* eggs were obtained from the Rainforest Reptile Refuge farm (Abbotsford, BC) and were used for the general morphology studies and radioactive in situ hybrdization. *P. regius* eggs were obtained from the Toronto Zoo and were used for dental explant organ culture experiments. *E. guttata* were obtained from colonies maintained at King's College London and the Stowers Institute (O. Pourquié lab) and used for slice cultures and whole-mount in situ hybridization.

Histological staining, PCNA staining and TUNEL reaction

Stage 6 and 8 python embryos and postnatal day 0 mice were demineralized in 5.5% EDTA with 4% paraformaldehyde for at least 2 months at room temperature prior to embedding in wax. Alternate slides were dual-stained with Picrosirius Red and Alcian Blue, which label bone and type I collagen-rich areas as red and cartilage (sulphated proteoglycans) as blue, respectively (Ashique et al., 2002).

PCNA (proliferating cell nuclear antigen) was detected immunohistochemically as described (Buchtová et al., 2007). TUNEL reaction was done with the ApopTag plus Peroxidase in situ Apoptosis Detection Kit (Chemicon, S7101) and sections counterstained with Methyl Green (0.1%).

3D reconstruction

Serial transverse sections were photographed and 3D image reconstruction was performed using WinSurf software (version 4.3, developed by Scott Lozanoff, University of Hawaii). The outer tooth row of the upper jaw was reconstructed in embryos at stages 3 (11 day), 4 (18 day) and 6 (33 day).

Cloning of African rock python Shh

Python tissues were snap-frozen in liquid nitrogen immediately after excision and stored at −80 °C until use. Total RNA was isolated from embryonic *P. sebae* head tissue (stage 3) using the Midi RNeasy Kit (Qiagen) according to manufacturer's instructions. First-strand cDNA was synthesized using Superscipt III (Invitrogen) and mRNA primed with oligo-dT (Invitrogen).

Forward and reverse degenerate primers against vertebrate orthologs of *Shh* (sense: 5′-ccggcttcgactgggtntayta-3′; antisense: 5′-catgggcggtcagtggngcrtangc-3′) were designed using the web-based design program CODEHOP and used to amplify a 502-bp fragment of African rock python *Shh* (*pShh*). This fragment, which we denote "pShh.1", was cloned into pGEM-T Easy (Promega) and used as a template to generate RNA probes (Fig. S1) for section in situ hybridization on *P. sebae* tissues. To confirm the orthology of the clone, we obtained additional sequence data, corresponding to the 5′ and medial segment of *pShh*, using these primers: 5′-gcttcaaggagctgaccccnaaytayaa-3′

(sense); 5′-acagagcagtgaatgtgcgcct-3′ (antisense). A contiguous transcript sequence of *pShh* was manually assembled from the 5′and 3′ sequence data, yielding an 888-bp segment of *pShh* (Fig. S1). This sequence has been deposited to NCBI GenBank (Accession #EU555185). Sequence contiguity was later confirmed by PCR with gene-specific primers, yielding a 774-bp cDNA clone of *pShh* ("pShh.2").

Sonic hedgehog orthology was confirmed by multiple protein sequence alignment and phylogenetic reconstruction with PHYLIP 3.66. The putative protein sequence of pShh shares 84% sequence identity with the *Gallus gallus* ortholog of Shh and >80% identity with other vertebrate orthologs (Fig. S2). Maximum-likelihood, parsimony and distance methods (UPGMA, Neighbor-joining) alike generated trees grouping the putative protein sequence of pShh (Fig. S3) with corresponding amniote orthologs and to the exclusion of closely-related paralogs (e.g., Desert and Indian hedgehogs).

In situ hybridization

Radioactive in situ hybridization (^{35}S-labelled UTP, GE Healthcare) on histological sections of African rock python (clone *pShh.1*) and mouse head tissues (mouse *Shh* provided by A. McMahon) were performed as described in Rowe et al. (1992). The *pShh.2* clone was used to generate digoxigenin-labeled probes for section in situ hybridization on ball python embryos and for whole-mount in situs on corn snake embryos. A hybridization temperature of 65 °C was used for both protocols. Non-radioactive section in situ hybridization was carried out as described (Wilhelm et al., 2007). For wholemount in situs on corn snakes, standard protocols were followed, including a 20-min pretreatment with proteinase K (10 μg/ml). Following hybridization, whole-mount embryos were post-fixed in 10% formaldehyde, embedded in 3.5% agarose and sectioned with a vibratome at a thickness of 50 μm.

Slice cultures of E. guttata mandibles

In order to visualise the development of the dental lamina, we adapted the technique of live slice culture of jaw tissues previously used to study odontogenesis in the mouse (Matalova et al., 2005). For this part of the project, we used corn snake embryos due to availability of freshly-laid eggs. Corn snake dental development closely follows that of the python (Bandali et al., unpublished observations). Embryos were collected 12 days after oviposition. At this stage the thickening of the dental lamina is just visible in the majority of embryos. Frontal slices 250 μm in thickness were made through the snake mandible of four embryos using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd. UK). The slices were separated out and the distal two-thirds of the mandible kept for culturing (3 slices per mandible). Slices were cultured on transparent Nucleopore filters supported on metal grids over medium. Medium consisted of DMEM supplemented with Pen/Strep, L-glutamine and 10% Fetal calf serum. A layer of Matrigel basement membrane (BD Bioscience) was placed on top of the slice to maintain morphology. Slices were cultured for up to 5 days in a CO_2 incubator and the same culture was photographed at Day 0 and Day 5. To half of the slices, 10 μM cyclopamine (Sigma) in DMSO was added to the medium and Matrigel, while the remaining control slices were given an equivalent concentration of DMSO. Affigel blue beads (Biorad) loaded with PBS were added to many of the slice cultures to aid orientation.

Organ culture of P. regius dental explants

Upper and lower jaws were excised from stage-6 heads of *P. regius*, hemisected, and then divided up into rostral and caudal segments. Right and left sides were paired and treated as controls or experimentals, thereby controlling for variation in tooth maturity between cultures and along the jaw. Experimental cultures were incubated in the presence of 10 μM cyclopamine (Toronto Research Biochemicals) dissolved in DMSO. Controls were treated with an equal volume of DMSO. Media was composed of DMEM: F12 (40:60), antibiotic/antimycotic (GIBCO), L-glutamine (GIBCO), abscorbic acid (50 μg/ml) and 10% Fetal Calf Serum. Organ cultures were placed on Nucleopore filters on top of wire mesh supports and cultured at the air–liquid interface in a CO_2 incubator. Following 5 days in culture, explants were embedded in paraffin and sectioned. Alternative sections were stained with Toluidine Blue, or used for PCNA or TUNEL staining.

Results

We previously described the development of the craniofacial complex of *P. sebae* in the context of a staging table, with stage 1 being coincident with oviposition and stage 8 with 54 days of incubation (Boughner et al., 2007; Buchtová et al., 2007). We use the same staging criteria in the present study. Tooth development begins after oviposition and thus we are able to study the full process from initiation to full crown formation. To gain a broader view of dental development in snakes, three species were chosen, two from the basal family Boidae (*P. sebae*, *P. regius*,) and one from a more derived family Colubridae (*E. guttata*; Vidal et al., 2007; Vidal and Hedges, 2002, 2005). Through comparison of these species, we define the conserved roles that *Shh* has in patterning the dentition in non-venomous snakes.

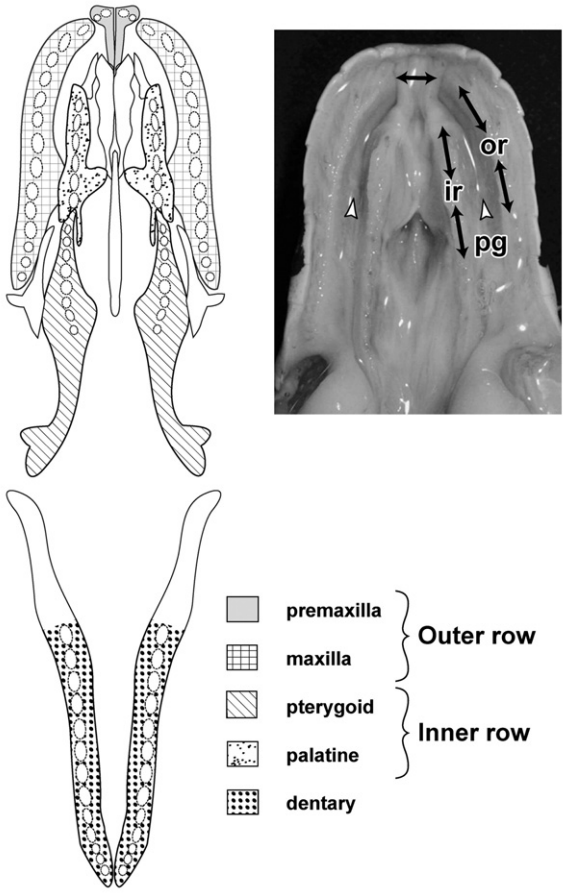


Fig. 1. Palatal (top drawing) and occlusal (lower drawing) view of the upper and lower jaw bones of pythons. Drawings of the adult python jaws in occlusal views. Palatal view of fresh specimen of a hatchling showing the palatine (inner row) and maxillary rows (outer row) of teeth separated by a palatal groove. This pattern is similar in all non-venomous snakes. Key: ir — inner row, or — outer row, arrowhead, pg — palatal groove.

Overview of snake tooth development

Unlike most lizards (Mahler and Kearney, 2006) and crocodilians (Westergaard and Ferguson, 1986), adult snakes have tooth rows lining both the margin and palate of the upper jaw. The outer or marginal row consists of the premaxillary teeth rostrally and maxillary teeth caudally; the teeth of these rows will later insert into the premaxilla and maxilla bones, respectively. Teeth of the inner row, called the palatine row, insert into the palatine and pterygoid bones (Fig. 1). Separating the marginal and palatine tooth rows and spanning the length of the upper jaw is a deep sulcus called the palatal groove (Fig. 1). In the lower jaw, a single row of teeth is attached to the dentary bone (i.e., dentary rows; Fig. 1). All the teeth of the snake are oral as opposed to a combination of oral and pharyngeal teeth seen in fish (Fraser et al., 2006a; Wise and Stock, 2006).

The maxillary, palatine and dentary teeth in pythons arise first from dental laminae. At stage 1, the earliest stage we collected of *P. sebae*, there are no morphological signs of any dental laminae except for a slight thickening in the maxillary row (Table S1 and Buchtová et al., 2007). The palatine and dentary rows are visible at stage 3 (Table S1, Figs. 2A,B). Based on the advanced development of the dentary rows, they would have been present earlier than stage 3, whereas the palatine row is initiating close to stage 3 in *P. sebae*. In later stages, development of the maxillary and dentary rows is largely synchronous, whereas the palatine row lags behind (Figs. 2D,E,G,H; Table S1). At stage 3, the dental laminae have formed in the centre of the jaws, but have not fully extended caudally or rostrally. Dentin is first

observed in some of the first generation teeth at stage 4 (Figs. 2D–F), while enamel spaces are first visible at stage 6 (data not shown). The palatal groove between the outer and inner rows becomes noticeable from stage 6 onwards in *P. sebae* (Figs. 2G,J).

The first generation teeth of *P. sebae* form deep within the mesenchyme (Fig. 2O; with the exception of premaxillary teeth, as discussed later). The second generation of teeth begins after the first generation reaches the cap stage (Figs. 2C,F). The third generation initiates by stage 6 (Fig. 2I) and up to 4 generations are visible in the stage 8 upper jaw (Fig. 2L, Table S1 and data not shown). The lower jaw forms a total of 3 generations of teeth by hatching. The first generation tooth maintains its connection to the dental lamina, and the dental lamina, in turn, remains connected to the oral epithelium up until at least stage 10 (Figs. 2J,K and data not shown). There is a characteristic orientation to the succedaneous lamina for each of the tooth rows, lingual for the maxillary and mandibular rows, labial for the palatine row. Tooth row orientation is an important focus in our subsequent organ culture experiments with explanted jaw tissues from the ball python *P. regius*.

Individual *P. sebae* teeth pass through largely comparable stages to those described for mouse and human teeth (Luckett, 1993; Tucker and Sharpe, 2004), including: (1) epithelial thickening (or initiation; Fig. 2M), (2) bud (Fig. 2O), (3) cap (Fig. 2P) and (4) bell stages (histodifferentiation, Fig. 2Q). There is an additional step in snake odontogenesis which we term the dental lamina stage (Fig. 2N). This stage is characterized by a finger-like projection of the dental epithelium into the mesenchyme with no signs of enamel organ formation (Fig. 2N). Another departure from the mammalian tooth mode is the simpler enamel organ of reptiles marked by a much reduced stellate reticulum (Fig. 2P), absent stratum intermedium, and lack of thickening of the inner enamel epithelium that could correspond to the mammalian enamel knot (Fig. 2P). At the cap stage, the succedaneous tooth lamina can be seen as a continuation of the outer enamel epithelium of the enamel organ (Fig. 2P). At the bell stage, formation of the dentin and enamel occurs in a similar manner to mammals, but at a much slower pace. It is possible to see teeth with just dentin and no enamel between stages 4 and 6. After the crown has formed there is no further development of the tooth and instead of roots, the cervical area of the crown attaches directly to bone (data not shown).

Mirror-image dental laminae form in the upper jaw

We noticed that the python dental laminae and associated mesenchyme are asymmetrically patterned in several respects. Firstly, palatal and maxillary laminae are each angled at 60° towards the palatal groove between them, appearing as mirror images of one another in transverse sections (Figs. 2A,D,G,J). Secondly, the mesenchyme also displays asymmetry: The extracellular matrix is richer in sulfated proteoglycans (as determined by Alcian Blue staining) on the side where the teeth are budding and is obtusely angled with the oral epithelium (Figs. 2D,E,G,H,J,K). The lower dental lamina most resembles the maxillary row in its angulation and extracellular matrix staining (Figs. 2B,E,H,K). These data predict that asymmetry in the distribution or activity of signaling molecules responsible for tooth initiation and differentiation may be present in snake teeth.

Dental laminae are continuous between maxillary, palatine and dentary teeth

Analysis of serial sections through the maxillary, palatine and dentary epithelia of *P. sebae* revealed that remarkably, all the sections, even those between teeth, contained a dental lamina. To obtain a more comprehensive view of the lamina and the positions of the teeth, we made three-dimensional (3D) reconstructions of the maxillary row at stages 3, 4 and 6 of *P. sebae*. The graphical output revealed several regularly spaced tooth anlagen along the ribbon-like dental lamina (Figs. 3A–G). At stage 3 and 4, the dental laminae are shorter at the rostral and caudal ends, whereas the middle part extends deeply into the mesenchyme (Figs. 3A–C, E–G and data not shown). From examining serial sections, we are certain that palatal and dentary dental laminae are also continuous (data not shown). The dental lamina makes an acute angle with respect to the oral epithelium and teeth arise on the obtusely angled side (Figs. 3B,E).

At stage 6, we observe that the crowns of the first generation teeth are nearly complete and that second generation teeth have formed sub-adjacently on the dental lamina (Figs. 3I–K). In addition to the acute angle of the dental lamina with respect to the oral cavity, we also noted another abrupt change in growth direction. Once the first generation teeth have formed, the lamina bends at a 90° angle as it proceeds to formation of the next generation (Figs. 3E–G,I–K). The bending of the dental lamina may be necessary to offset the different generations of teeth thus providing the necessary space to complete morphogenesis.

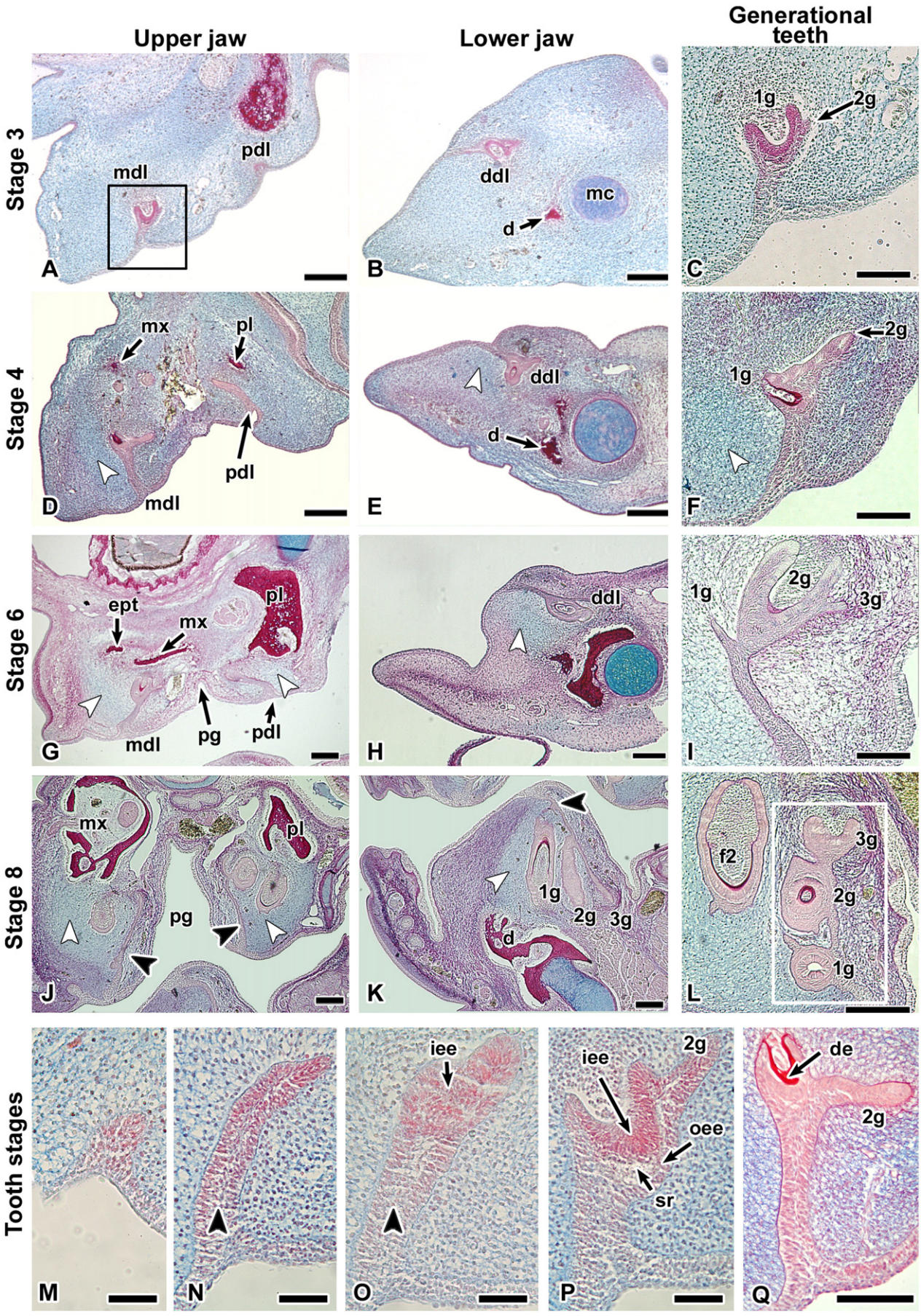
Shh expression is localized first to the odontogenic band and then in the inner enamel epithelium

We hypothesized that the many interesting characteristics of the *P. sebae* dental laminae such as the medial–lateral asymmetry of the upper rows could be due to asymmetrical gene expression patterns, cell proliferation and/or apoptosis. Our aim was to look for 'handedness' in molecular markers that may precede the asymmetry of morphogenesis.

We chose to survey the expression of *Shh* since it is one of the earliest signals expressed in the vertebrate dental epithelium (Hardcastle et al., 1998). Blocking Shh signaling in mouse arrests tooth development at the initiation stage (Cobourne et al., 2001), and once teeth have initiated, Shh is required for patterning of the teeth and histodifferentiation of enamel (Dassule et al., 2000). We chose to survey *Shh* expression in members of a basal snake family, the Boidae (*P. sebae* and *P. regius*) and a more derived family, the Colubridae (*E. guttata*; Vidal et al., 2007; Vidal and Hedges, 2005), to explore the conservation of expression within the non-venomous snake taxa. The description that follows deals primarily with the two python species, for which we had the most comprehensive stage series.

As in other vertebrates, snake *Shh* is restricted to the epithelium (Figs. 4A–H), notochord and ventral floorplate of the neural tube of *P. sebae* (data not shown). These patterns are specific to *Shh.* There was no signal in endochondral bone of the vertebrae or intramembranous bones of the face areas where Indian hedgehog is strongly expressed (Rice et al., 2006). The data demonstrate that our probe does not cross-hybridize with other members of the hedgehog family. In addition our probe cross reacts with 2 other snake species used in this study, suggesting high conservation of coding sequences. Expression is present in the oral epithelium at stage 1 in *P. sebae* even prior to thickening of the future dental lamina in the lower row (Figs. 4A,B). There is no expression of *Shh* in the frontonasal mass at stage 1 (data not shown). In contrast, *E. guttata* has expression in the frontonasal mass (Figs. 5A,C), however these differences could be related to slight differences in developmental stage of the embryos. Elsewhere in the oral cavity, *E. guttata* has identical *Shh* expression in the presumptive odontogenic band (Figs. 5B,D,E) to that seen in the stage 1 *P. sebae*.

At stage 3, *Shh* is strongly expressed at the junction of the oral and dental epithelia on the acutely-angled side of the dental laminae (Figs. 4E,F). The identical pattern is seen in *P. regius* embryos (Fig. 4G). This oral ectodermal expression domain spans the length of the dental lamina along each jaw at stages 3 and 4


Upper jaw
Lower jaw
Generational teeth
Stage 3
Stage 4
Stage 6
Stage 8
Tooth stages
mdl
pdl
ddl
mc
d
1g
2g
mx
pl
ept
pg
3g
f2
iee
oee
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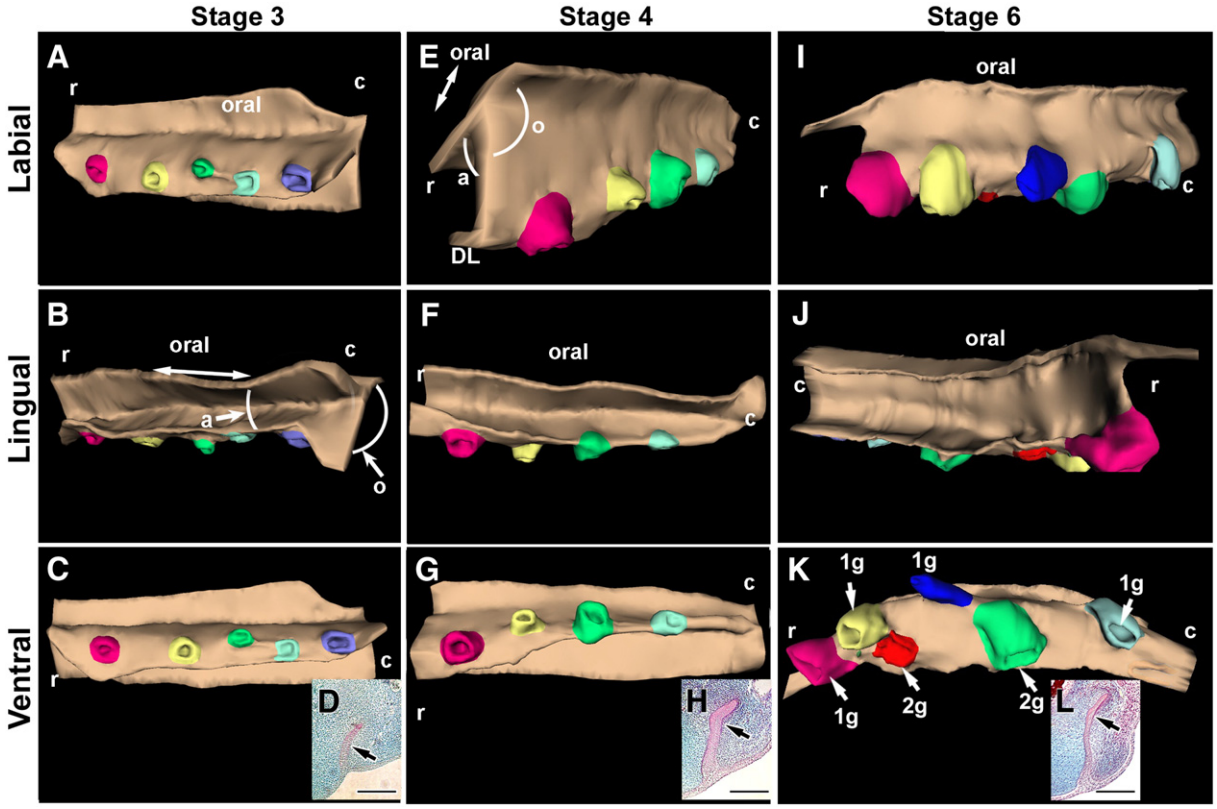

Fig. 3. Continuity of the maxillary dental lamina in *P. sebae*. Reconstruction of the maxillary dental lamina. There are no gaps between sections and only epithelium was traced. Examples of sections traced are in panels D,H,L. (A–C) 1840 μm was reconstructed. The dental lamina is continuous and cap stage primary or first generation teeth are present at regular intervals. The angle of the dental lamina with respect to the oral epithelium is indicated in panel B. (E–G) 1440 μm was reconstructed. The tip of the dental lamina is bent at 90° to the rest of the dental lamina. First generation bell stage teeth bud off the labial side or obtusely angled side of the lamina (E,F). (I–K) 1197 μm was traced. Several tooth families (first and second generation) can be seen. The pink, green, yellow and dark blue anlagen are bell-stage, whereas the red and turquoise teeth are cap stage. The second-generation teeth are closest to the growing tip of the dental lamina. The dental lamina has an S shape (I,J). Key: a — acute angle, c — caudal, DL — dental lamina, o - obtuse angle, r — rostral, 1g — first generation tooth, 2g — second generation tooth. The reconstructions are not to scale. Scale bar for sections = 200 μm.

(Fig. S4A–R) and thus *Shh* delineates the position of the odontogenic epithelium in the facial prominences. Within the enamel organs of cap stage teeth, *Shh* expression is localized to the inner enamel epithelium (Figs. 4D,F,H; Fig. S4C,D,K,L). It is important to note that *Shh* expression in the enamel organ is neither temporally or spatially continuous with the expression domain in the oral epithelium. In addition to dental expression, *Shh* transcripts are seen in the nasal orifice epithelium, vomernasal cushions, dorsum of the tongue and tongue sulcus for both species of python (Figs. 4C–F and data not shown). At stage 6 in *P. sebae* and *P. regius*, the oral expression is much more restricted (Fig. 4G) and, at some levels along the jaw, the expression domain has been lost altogether (Fig. S5A–F). Within the teeth, *Shh* is lost from the centre of the inner enamel epithelium while transcripts are visible for the first time in the stellate reticulum (Figs. 4H,I; Fig. S5B). During the formation of generational teeth, there is no expression in the extending dental lamina (Fig. 4I) until a tooth begins to form (Figs. 4F,H). Overall, the expression of *Shh* in the two python species was identical as was the general patterning of the craniofacial tissues.

We noted several differences in *Shh* expression between developing mouse and python teeth. Specifically, expression in the mouse

Fig. 2. Dental development in the African rock python *Python sebae*. Transverse (frontal) sections stained with Picrosirius Red, Alcian Blue. (A,D,G,J) The upper dental laminae are inclined towards the palatal groove, in a mirror image configuration, and are situated close to the maxillary and palatine bones. The maxillary dental lamina is more advanced than the palatine lamina and teeth form on the obtusely angled side of the dental laminae. (C) A higher magnification of the cap stage tooth in panel A showing the dental lamina that will form the second generation. The extracellular matrix on the obtusely angled side of the dental laminae is richer in sulfated proteoglycans and is therefore stained with Alcian Blue (white arrowheads). (J,K) The connection between the dental lamina and the oral ectoderm is still robust and showing no signs of degradation (black arrowheads). (B,E,H,K) The dentary lamina is sharply angled towards the dentary bone and Meckel's cartilage. Similar to the upper teeth extracellular matrix is more sulfated on the obtusely angled, tooth-forming side of the dental lamina (white arrowheads). (K) There is very little space between the dental lamina with three sets of teeth and the tongue sulcus. (C,F,I,L) The progression of generational tooth formation from the earliest bud to the most advanced with multiple generations being present. Note how the generational lamina emerges from the outer layer of the first generation tooth bud. In panel I, the first generation tooth is out of the plane of section but 2nd and 3rd can be seen. (L) Three generations of a single family (white box). The fourth generation tooth germ is not visible in this section, however a tooth from a different family is present nearby. (M–Q) Stages of tooth formation in the snake. (M) Initiation stage — there is a short thickening of the dental epithelium. (N) Dental lamina stage — elongation of dental epithelium in maxillary, palatine and dentary tooth rows. This stage is not present in the premaxillary teeth. Black arrowhead points to middle layer of cells sandwiched between two outer layers that are anchored to the basement membrane. (O) Bud stage — thickening of the tip of the dental lamina and formation of the inner enamel epithelium. The three layers of the dental lamina are still visible (black arrowhead shows middle layer). (P) Cap stage — The three layers of the tooth germ are distinct. Note that the inner enamel epithelium curves around the dental papilla. The lamina that will form the second generation tooth arises from the outer enamel epithelium. The stratum intermedium is derived from the middle layer of the dental lamina. (Q) Bell stage — Differentiation of dentin from the inner enamel epithelium has begun in the first generation tooth. Key: d — dentary bone, ddl — dentary dental lamina, de — dentin, ept — ectopterygoid, f2 — a tooth from a different tooth family, iee — inner enamel epithelium, mc — Meckel's cartilage, mdl — maxillary dental lamina, mx — maxillary bone, oee — outer enamel epithelium, pdl — palatine dental lamina, pg — palatal groove, pl — palatine bone, sr — stellate reticulum, 1 g — first generation tooth, 2 g — second generation tooth, 3g — third generation tooth, 4g — fourth generation tooth. Scale bars for panels A,B,D,E,F,H,J,K = 200 μm; C,F,I,L,Q = 100 μm; M–P = 50 μm.

enamel organ is restricted to the inner enamel epithelium and never extends into the stellate reticulum (Figs. 4J–M). In addition, there is no oral expression adjacent to the dental epithelium in the mouse (Figs. 4J,K,M; Rice et al., 2006; Sarkar et al., 2000). The closest oral epithelial expression to the teeth is seen in the groove lateral to the palatal shelves (Fig. 4J).

Asymmetrical gene expression and proliferation precedes directed morphogenesis

In order to determine whether Shh could be acting to promote morphogenesis of the snake tooth, we examined cell proliferation and apoptosis and correlated these data with sites of *Shh* expression in *P. sebae*. In the initiating maxillary dental lamina, *Shh* is restricted to the medial side of the epithelial thickening (Fig. 6A) precisely overlying the PCNA-positive mesenchyme (Fig. 6F). The data from these early stages of tooth morphogenesis, when the dental lamina is still overtly symmetrical, reveal an unexpected degree of 'handedness' in the mesenchyme (i.e., PCNA staining) and in the epithelium (i.e., *Shh* expression).

In contrast to the mesenchyme, areas of epithelium expressing *Shh* had relatively lower cell proliferation (Figs. 6C–E,H–J). When the dental lamina is just beginning to extend into the mesenchyme, the dental lamina is trilaminar with two outer, polarized layers attached to the basement membrane and a middle layer of loosely packed epithelial cells. At first, symmetrical PCNA staining is found in both outer layers of the invaginating dental lamina (Fig. 6G), but later when teeth form, proliferation is mainly found on the obtusely-angled side of the lamina, opposite to where *Shh* expressed (Figs. 6C,H). These data suggest bending of the entire dental lamina (Figs. 3A–K) is a result of this differential proliferation within the epithelium. Thus Shh protein does not appear to act in a cell-autonomous way to stimulate proliferation. Rather, the effects are seen to diffuse a short distance away and possibly stimulate proliferation in adjacent epithelial and mesenchymal cells (Figs. 6G,J).

In contrast to the distinctly asymmetrical patterns of cell proliferation and *Shh* expression, apoptosis around and within the dental lamina was less patterned. Several apoptotic cells were present in the epithelium in the middle layer of the dental lamina (Fig. 6M). In the mesenchyme, the apoptotic cells in the mesenchyme were associated with mesenchymal condensations for intramembranous bones and not the teeth (Figs. 6K,L; Buchtová et al., 2007).

The replacement teeth recapitulate the patterns of apoptosis, cell proliferation and *Shh* expression as seen in the first generation teeth (Figs. 4F,H; Figs. 6E,J,O). Furthermore, we saw no differences in proliferation, apoptosis and gene expression in the dental lamina at any level along the jaw, suggesting that tooth-forming capacity is generalized within the structure (data not shown). The delineation of tooth-forming and inter-dental regions in the dental epithelium instead appears to be specified by signals originating in the adjacent mesenchyme and not by intrinsic differences in the ectoderm.

Evaluation of enamel knot markers in the inner enamel epithelium of python

The enamel knot of the mouse molar is a histologically distinct thickening of the inner enamel epithelium characterized by low cell proliferation, restricted expression of *Shh* and high apoptosis. In *P. sebae*, we could not find a thickening in the inner enamel

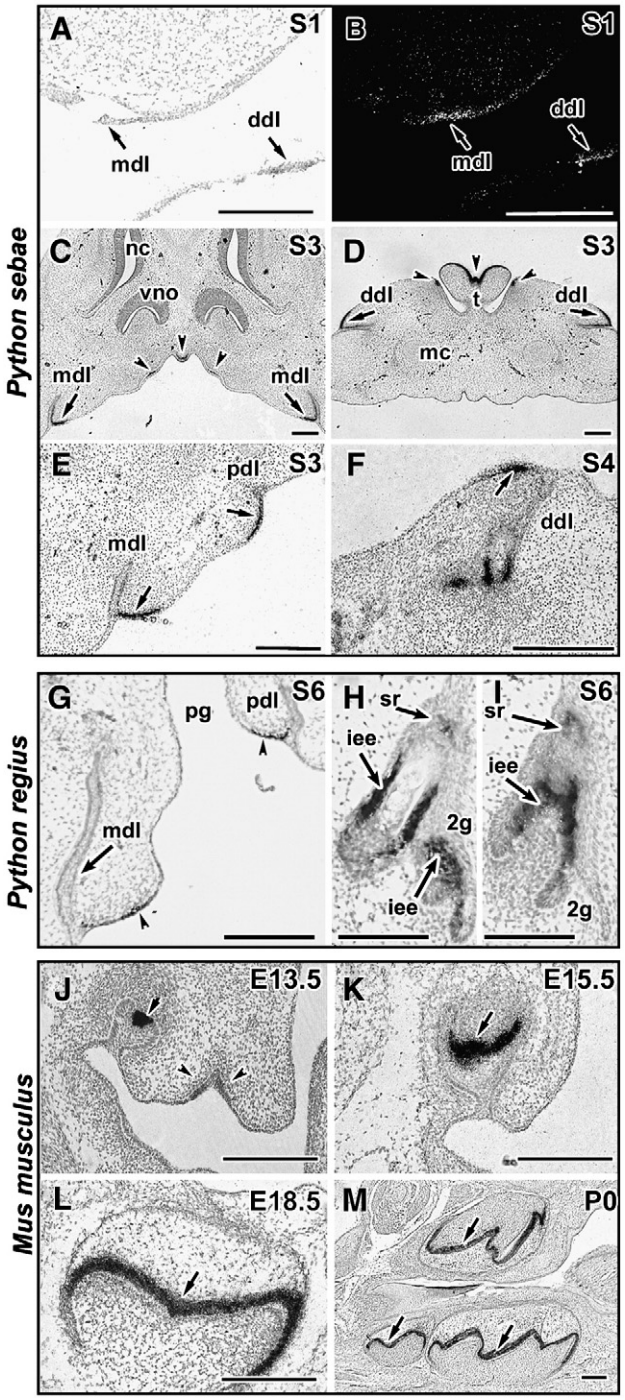

Fig. 4. A comparison of *Shh* expression during dental and craniofacial development of two *Python* species and mouse. Radioactive probe on *P. sebae* (A–F), digoxygenin probe on *P. regius* (G–I), and radioactive probe on *Mus musculus* (J–M). (A,B) *Shh* expression is seen in the maxillary dental lamina and the presumptive dentary lamina (signal can only be detected in darkfield, panel B). (C, D) Expression elsewhere in the oral cavity is in the epithelial folds beneath the vomeronasal organ, the crest of the lingual sulcus and centre of the tongue (arrowheads). (E) Mirror-image expression domains in the upper jaw. Initially, expression in the oral ectoderm extends part way down the dental lamina (arrows). (F) Expression is maintained in the oral–dental epithelial interface and is also initiated in the presumptive inner enamel epithelium of second generation teeth; however, there is no connection between the tooth expression and that seen in the oral ectoderm. (G) Oral epithelial expression of *Shh* (arrowheads) on the acutely-angled side of the maxillary and palatal dental laminae is identical to *P. sebae* (E). (H) *Shh* transcripts in the inner enamel epithelium of a first-generation bell-stage tooth. Expression can also be seen in the stellate reticulum. The second-generation tooth is also expressing *Shh* in the inner enamel epithelium. (I) *Shh* is expressed within the enamel organ (inner enamel epithelium and stellate reticulum), but not in the successional rudiment. In the mouse, *Shh* transcripts are strongly focused in the enamel knot (arrow in panel J) and palatal groove (arrowheads). Expression expands within the inner enamel epithelium (K) to including the ameloblasts but is absent in the stellate reticulum (L, M). Key: ddl — dentary dental lamina, iee — inner enamel epithelium, mc — Meckel's cartilage, mdl — maxillary dental lamina, pg - palatal groove, nc — nasal conchae, pdl — palatine dental lamina, sr — stellate reticulum, t — tongue, vno — vomeronasal organ, 1 g — first generation tooth anlagen, 2 g — second generation tooth anlagen. Scale bar = 200 μm for all except H,I = 100 μm.

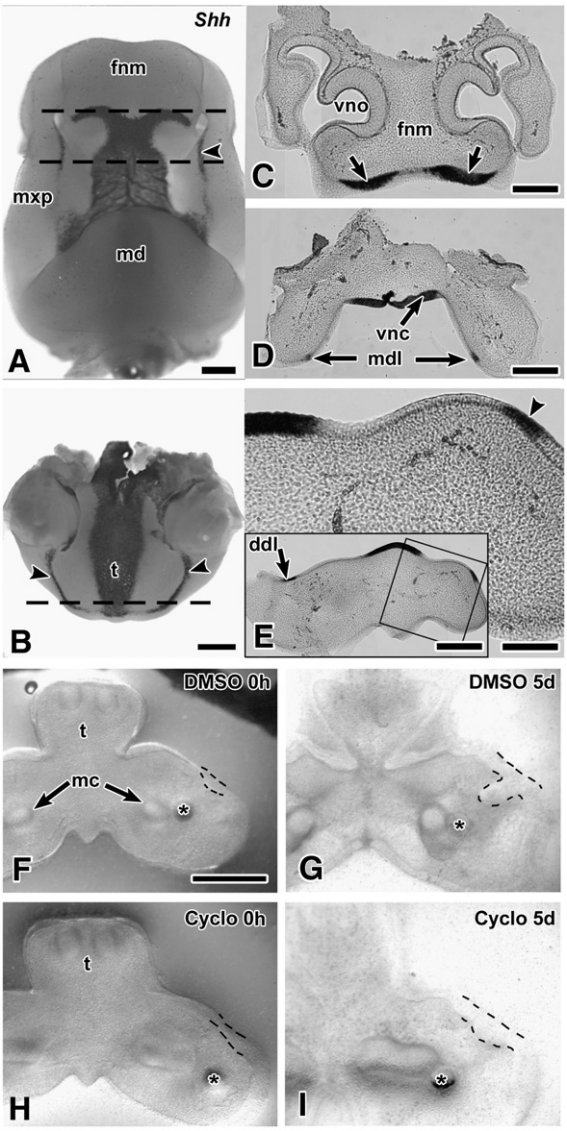

Fig. 5. Expression of *Shh* and functional blocking of Hh signaling in *Elaphe guttata* embryos. (A–E) Whole-mount in situ hybridization of E6 (stage 1–2) corn snake embryos. (A) Frontal view of the snout showing expression at the edge of the frontonasal mass is continuous with expression in the roof of the oral cavity. A distinct band of *Shh* signal is continuous along the maxillary prominences (arrowhead). (B) Superior or dorsal view of the lower jaw cut away from the head. The flat tongue primordium expresses high levels of *Shh* as does the dentary dental lamina (arrowheads). (C) Vibratome section through the frontonasal mass showing epithelial expression (arrows). (D) Section through the maxillary prominences showing maxillary dental laminae and expression in the vomeronasal cushions. (E) Vibratome section of embryo in panel B showing no thickening of the dentary dental lamina (arrowhead). Lower power inset shows bilateral expression in dentary dental lamina in the future tongue. (F–I) E12 (stage 2) corn snake embryo slices 250 μm thick placed into organ culture and photographed serially as unstained preparations. (F,H) Slight thickening is present at Day 0. Site of future dental lamina (dashed lines) based on appearance of the same cultures 5 days later (G,I). A dental lamina has formed in the control (G) but not in the experimental culture (I). Affigel blue beads are inserted for orientation purposes (asterisks). Key: ddl — dentary dental lamina, fnm — frontonasal mass, mc — Meckel's cartilage, md — mandibular prominence, mdl — maxillary dental lamina, mxp — maxillary prominence, t — tongue, vnc — vomeronasal cushions, vno — vomeronasal organ. Scale bar = 300 μm; bar for high power view in panel E = 100 μm.

epithelium corresponding to an enamel knot. The entire inner enamel epithelium, however, shares features in common with the mammalian knot, specifically low or absent proliferation (Figs. 6I,J) and high levels of *Shh* transcripts (Figs. 4F,H,I; 6D,E). Cell apoptosis is not seen in the inner enamel epithelium. Instead, we noted a cluster of TUNEL-positive cells in the stellate reticulum (Figs. 6N,O), overlapping with high *Shh* expression (compare to Figs. 4H,I). There is also a discrete cluster of apoptotic cells in the dental papilla (Fig. 6N and data not shown).

Hedgehog signaling is required to set up directionality and to promote elongation of the dental lamina

The dental expression of *Shh* suggests that there may be requirements for Shh signaling in the 1) induction of the dental lamina, 2) directionality of tooth formation, 3) extension and generation of the generational lamina, and 4) maintenance of the inner enamel epithelium and stellate reticulum. To address these hypotheses, we treated snake jaw explants with cyclopamine, a specific antagonist of hedgehog signaling (Chen et al., 2002) at initiation stages and once teeth had formed. While we acknowledge that cyclopamine treatment will potentially affect all hedgehog signaling, there is no evidence from mouse studies that *Desert* or *Indian hedgehog* will be expressed in dental tissues (Bitgood and McMahon, 1995). Furthermore, the diffusible nature of Shh protein (Chamberlain et al., 2008) suggests that tissues adjacent to the oral epithelium and enamel organ will be affected.

Inhibition of Hh *signaling* in day 12 corn snake cultures led to a complete block in dental lamina ingrowth. Only a slight epithelial thickening was observed in the experimentals after 5 days in culture (compared to $n=4/4$; Fig. 5I), practically unchanged in size from the outset. In contrast, control cultures showed finger-like dental laminae that had extended considerably into the dental mesenchyme during the culture period ($n=6$; Fig. 5G).

In the stage 6 *P. regius* control cultures, maxillary and dentary dental laminae appeared sharply angled with first and second generation teeth forming deep in the mesenchyme after 5 days in culture (Figs. 7D,J and 8A–C). PCNA labeling in these cultures revealed cell proliferation in the basal layer of the oral epithelium and at the tip of the dental lamina on its tooth-budding side (Figs. 7F,F′L,L′). Apoptosis is instead mostly confined to the cut edges of the explants and around the ossifying bones (Figs. 7B,E,H,K). Differentiation of the enamel organ in culture was similar to that observed in sections of stage 6 python embryos (Figs. 7J; 8A′,B′,C′). Overall, culturing the tissues did not have detrimental effects on cellular dynamics.

The effects of cyclopamine in the *P. regius* organ cultures were most noticeable in the dental lamina. There was an obvious reduction in the length of dental laminae as compared to paired, control cultures (Figs. 7A–C; G–I). Furthermore, the distinct acute angle of the dental lamina is lost in the cyclopamine-treated cultures (Figs. 7A,G; 8D-F) and instead the lamina is oriented almost at 90° to the oral epithelium (Table 1). In some cultures the teeth have become very superficial relative to the oral ectoderm (Figs. 8D–F). There was increased mesenchymal apoptosis in comparison to DMSO controls and the majority of the apoptotic cells were adjacent to the oral ectoderm (Figs. 7B,H). Cyclopamine did not block proliferation as PCNA-positive cells were seen throughout the treated cultures; however, PCNA staining did appear to be more symmetrical within the cyclopamine-treated dental laminae (Figs. 7C′,J′) than in the DMSO-treated cultures (Figs. 7F,F′) or *in vivo*. The lamina for the second generation teeth is still evident in the cultures treated with cyclopamine (Table 1), but is sometimes oriented in the opposite direction in relation to the maxillary bone (Figs. 8D,D′). The same effect is observed in mandibular teeth where the second-generation lamina is directed away from (Figs. 8F, F′) as opposed to towards the dentary bone (Figs. 8C,C′). It could be that the abnormally shaped first generation tooth affected the patterning of the generational lamina. Within the majority of enamel organs seen in the cultures, the stellate reticulum appears cystic and disorganized and the inner enamel epithelium thinner compared to the controls (Table 1; Figs. 8D′–F′ compared to A′–C′). Among those second generation anlagen that formed during the culture period, the enamel organ appears as little more than an

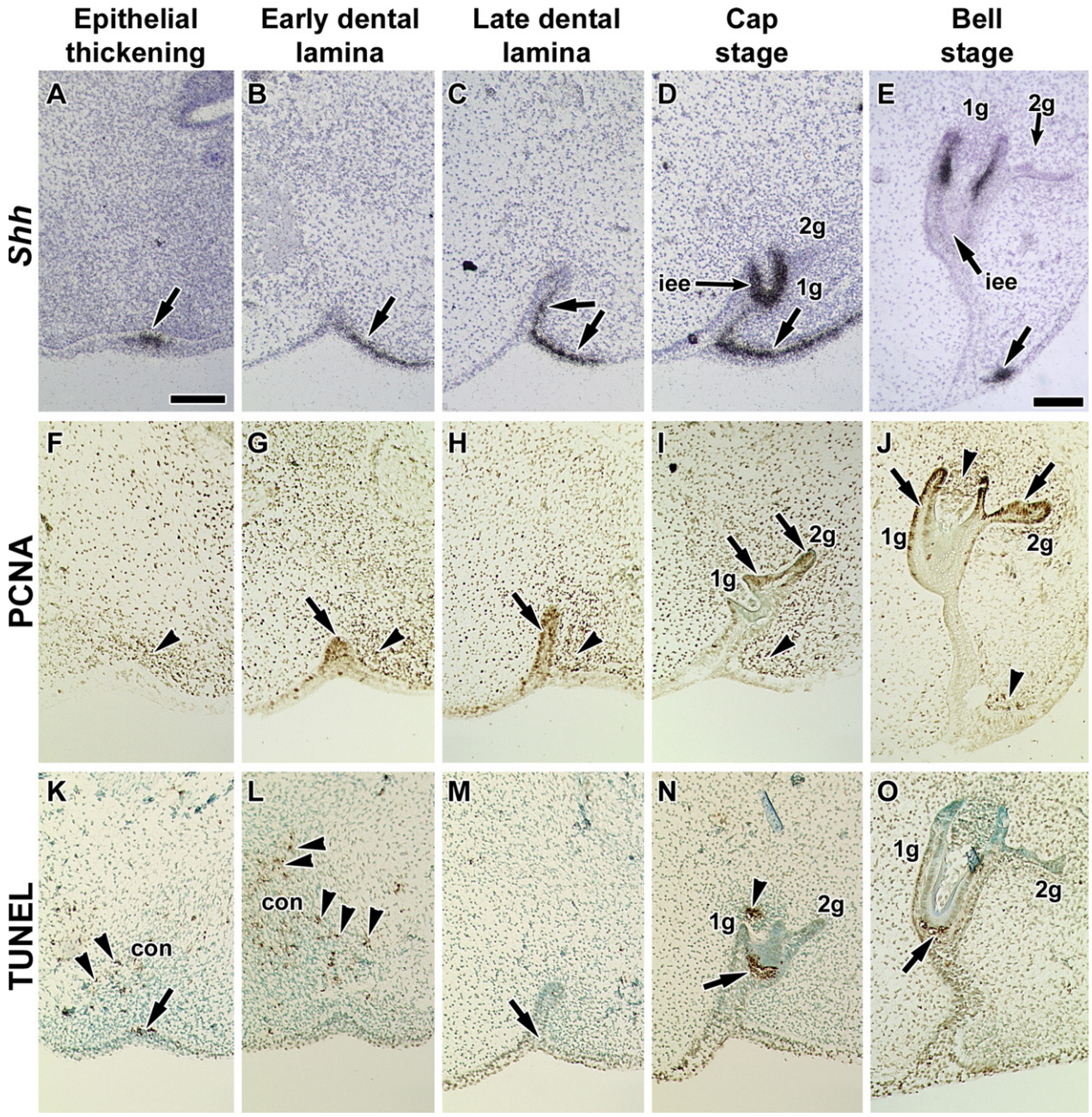

Fig. 6. *Shh* expression in the African rock python *Python sebae* overlaps with areas of low proliferation, but not with areas undergoing apoptosis. Near adjacent, transverse sections through the maxillary dental lamina. (A–E) Strong expression on one side of the dental lamina and within the inner enamel epithelium (arrows). Signal in parts of the inner enamel epithelium is already receding panel E. (F–J) Epithelial proliferation is highest (arrows) in areas that do not express *Shh* including the cervical loop and tip of the dental lamina. Proliferation throughout the inner enamel epithelium is low (I,J). Mesenchymal cell proliferation is highest close to the oral ectoderm that expresses *Shh* (black arrowheads in panels F–J). (K,L) Apoptotic cells are seen in the pre-osseous mesenchymal condensation (arrowheads) and in the surface ectoderm (arrow). (M) Apoptotic cells in the central dental lamina (arrow) from which the stellate reticulum arises. (N,O) Apoptosis in the stellate reticulum (arrows) and in the dental papilla (arrowhead in panel N). Key: 1 g — first generation tooth anlagen, 2 g — second generation tooth anlagen, con — mesenchymal condensation, iee — inner enamel epithelium. Scale bar = 100 μm.

epithelial cup, lacking clear histodifferentiation of the inner enamel epithelium and stellate reticulum (Figs. 8E,E′). Collectively, the effects of cyclopamine are directed to all the areas where *Shh* expression is observed *in vivo*.

Premaxillary teeth are formed superficially and do not have Shh expressed in the oral ectoderm

The greatly shortened dental laminae in cyclopamine treated cultures suggested that there might be a connection between oral ectodermal *Shh* and the depth of the dental lamina. We therefore looked at *Shh* in a region of the mouth where teeth are much more superficial, the premaxilla. In stage 3 *P. regius* embryos (Figs. 9A–C), there was expression of *Shh* in the rostral part of the oral ectoderm (Figs. 9A,B) and where premaxillary teeth were forming there was expression in inner enamel epithelium (Figs. 9A′,B). The dental lamina did not express *Shh* (Figs. 9A′). More caudal sections show the expression of Shh in the stomodeal epithelium (Figs. 9B) similar to that seen in *E. guttata* (Figs. 5A,D). Progressing more caudally, the oral ectoderm had very weak *Shh* signal whereas the egg tooth and premaxillary dental lamina had stronger expression (Figs. 9C,C′). This oral ectodermal expression is completely lost by embryonic stage 4 (Figs. 9D,D′) and 6 embryos (Figs. 9E,E′). The inner enamel epithelium of premaxillary teeth expresses *Shh* as seen elsewhere in the mouth

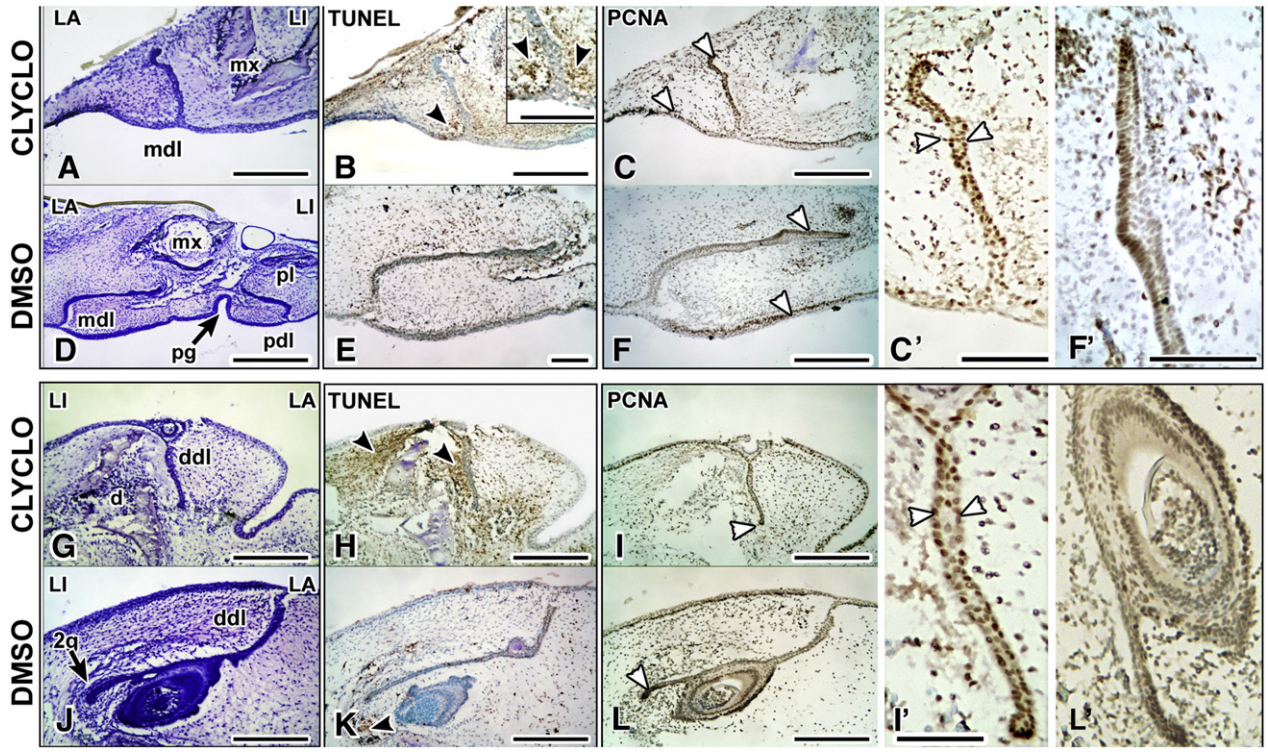

Fig. 7. Effects of reduced Hh signaling on the ingrowth of the dental epithelium, tooth morphogenesis, and the directionality of tooth replacement in *Python regius*. Transverse sections through explanted dental tissues from the upper and lower tooth rows cultured for 5 days in the presence of 10 μM cyclopamine or DMSO. Jaws were hemisected and the contralateral sides used as experimental and control cultures, thereby controlling for differences in initial tooth maturity between specimens. (A–C, G–I) In cyclopamine-treated cultures, dental laminae appear shorter and more perpendicularly-oriented with the oral epithelium compared to DMSO-treated controls (D–F, J–L). There was an increase in apoptosis in the mesenchyme (black arrowheads in panels B, H and inset in panel B); however, cell proliferation (white arrowheads) appears more evenly distributed on both sides of the cyclopamine-treated laminae (C′, I′) than in the controls (F′, L′). Key: 2 g — second generation, cyclo — cyclopamine, d — dentary bone, ddl - dentary dental lamina, iee — inner enamel epithelium, LA — labial, LI — lingual, mdl — maxillary dental lamina, mx — maxillary bone, pdl — palatal dental lamina, pg — palatine groove, pl — palatine bone. Scale bar = 200 μm, 100 μm for insets.

(Figs. 9E,E′ and data not shown). Thus, pythons have two distinct types of dental development that appear to be dependent on the degree to which *Shh* expression is maintained in the oral epithelium.

Discussion

Here we provide the first molecular and functional analyses of odontogenesis in a snake. Our data reveal surprising asymmetries in the snake dentition at all levels of biological organization. At the histological level, the dentition displays a dramatic angulation with the oral ectoderm, with primary and generational teeth forming exclusively on one side. At the cellular level, proliferating cells are also found only on this side of the dental lamina, where they provide the material for nascent tooth anlagen and promote the directional growth. Finally, at the molecular level, we noted expression of the gene *Sonic hedgehog* only on the non-tooth-budding side of the lamina, where it acts remotely to establish the overt asymmetry of the snake dentition. We present data here that specifically implicates Hh signaling in the initiation and directional ingrowth of the dental lamina, the asymmetric budding of tooth anlagen, as well as in the subsequent growth and maintenance of the enamel organ.

Evolutionaryary conservation of early Shh expression in odontogenic band of dentate vertebrates

In the youngest snake embryos collected for this study (*P. sebae*, stage 1; *E. guttata*, stage 2), we see a continuous band of *Shh* expression along the length of the upper and lower jaws that precedes epithelial thickening and establishment of the dental lamina. Thus it is likely that in snakes *Shh* is used to position the dental lamina within the ectoderm of the facial prominences (Fig. 10A). Once expression begins, the epithelium becomes committed to forming dental lamina. As our corn snake slice-culture data clearly shows, *Shh* is also necessary for ingrowth of the dental lamina into the mesenchyme.

How widespread is the presence of a *Shh*-expressing odontogenic band in gnathostomes? Recent work has revealed a similar expression pattern of *Shh* in the oral ectoderm of both trout (Fraser et al., 2006a,b, 2004) and alligator embryos (Harris et al., 2006). In some mammals, such as rodents, there are two discrete foci of *Shh* expression rather than a continuous band as we have described in snakes. These two domains are most apparent in the mandibular arch where there are no gaps between facial prominences (Cobourne et al., 2004). However, rodents are unique amongst mammals since they lack both canines and premolars and instead have a diastema region. In mammals with all the tooth types (e.g., Primata, Carnivora), there is predicted to be a more continuous band of expression. Indeed, recent data from the shrew show this to be true (Miyado et al., 2007). The relationship of the odontogenic band to sites of dental lamina invagination was revealed in our cyclopamine-treated *E. guttata* cultures, which demonstrated a complete lack of invagination of the lamina. Thus we are confident that Hh signaling is necessary for these early steps of dental epithelial commitment (Figs. 10A,B). Functional tests have not yet been carried out in fish teeth, but it is likely that *Shh* will play a similar pivotal role in positioning the dental lamina and triggering invagination.

Maintenance of Shh in the oral ectoderm is required for continued growth and positioning of the dental lamina

In the python, *Shh* is expressed in the oral ectoderm adjacent to the dental lamina in the maxillary, dentary and palatal tooth rows. This expression domain likely represents a remnant of the earlier odontogenic band. It is possible that the continued oral expression

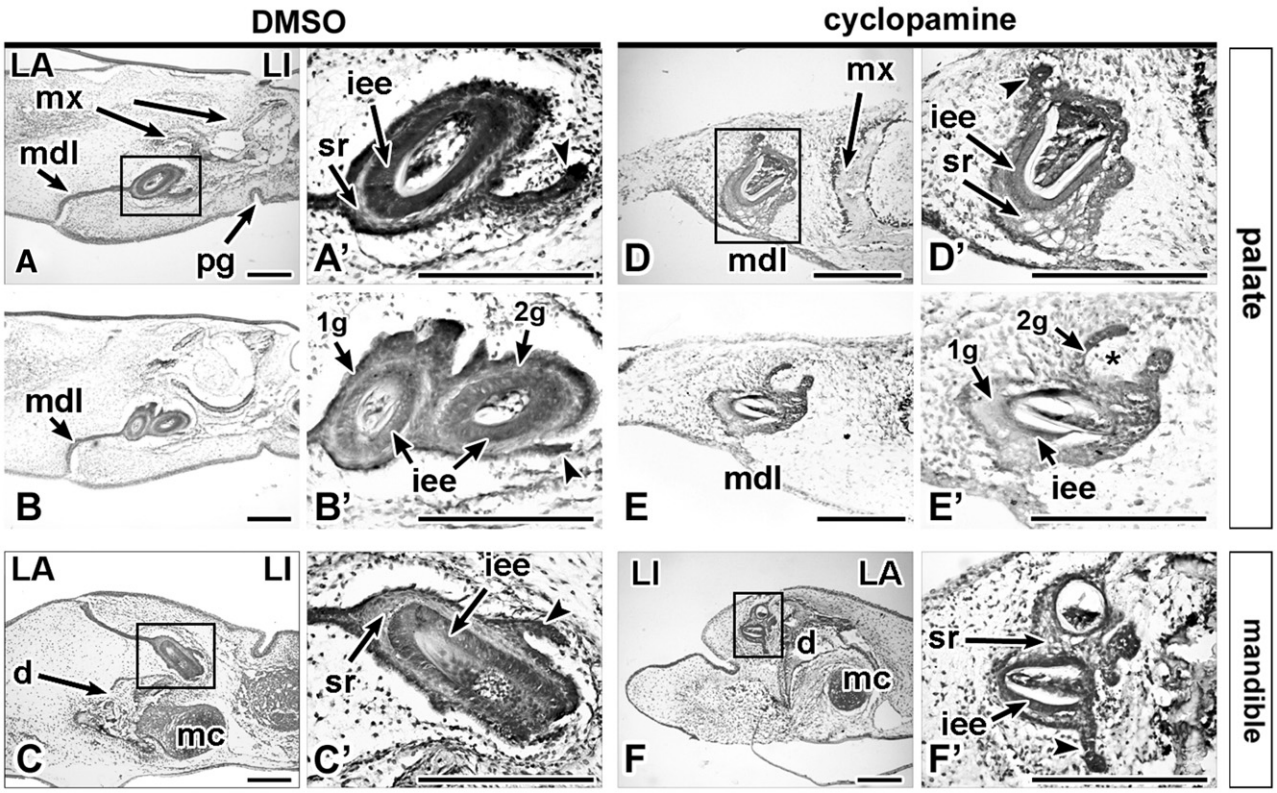

Fig. 8. Effects of decreased Hh signaling on the enamel organ and the directionality of generational dental lamina in *Python regius*. Stage 6 *P. regius* jaw tissues cultured in the presence of 10 μM cyclopamine or DMSO for 5 days. Transverse sections through dental explants (A–B) are sections all from the same culture (D,E) from the same culture. (A′–C′) Control cultures have first generation teeth and second or third generation laminae (black arrowheads). Stellate reticulum and inner enamel epithelium are normal. (D–F) Cyclopamine-treated tooth germs show cystic stellate reticulum as well as thinner inner enamel epithelium. The generational lamina (black arrowheads) is on the opposite side of the tooth germ in the treated cultures (compare panel D′ with panel A′, panel C′ with panel F′). (E′) Second-generation teeth formed in the presence of cyclopamine appear smaller in size and lack a clearly identifiable inner enamel epithelium and stellate reticulum compared to control-treated teeth. Key: 1 g — first generation tooth anlagen, 2 g — second generation tooth anlagen, d — dentary bone, iee — inner enamel epithelium, LA — labial, LI — lingual, mc — Meckel's cartilage, mdl — maxillary dental lamina, mx — maxillary bone, pg — palatal groove, sr — stellate reticulum. Scale bar = 200 μm.

of *Shh* helps to promote lengthening of the dental lamina. Our cyclopamine data is in agreement with this idea: We consistently observed shorter dental laminae (Fig. 10C) and tooth anlagen that were located at shallower depths in the dental mesenchyme. Premature down-regulation of *Shh* in the dental epithelium of the mouse has also been shown to affect the depth of tooth-budding (Dassule et al., 2000). In the conditional K14 knock-out mouse, teeth completely lack a dental cord and are essentially fused with the oral epithelium. Shh may then have an evolutionarily conserved function in promoting distal tooth-budding in amniotes.

Since there is no *Shh* expression within the dental lamina itself, it is likely secreted SHH protein from either the oral ectoderm or the enamel organ that instructs its growth. By this scenario, cyclopamine blocks the normal activity of the Smoothened (Smo) transmembrane receptor in the dental lamina (Chen et al., 2002), rendering it unresponsive to the inductive signal and causing stunted, direction-less ingrowth. Hedgehog-responsiveness of the dental lamina could be revealed by hybridizing probes against *Smo* and downstream Hh transmembrane protein *Patched*. These expression data would also clarify the role of Hh signaling in generational tooth formation. The presence of *Smo* or *Ptc* transcripts in the outer enamel epithelium and/or successional lamina would indicate that the tissue is responding to SHH signal. Our culture data suggest that there may be a role for Shh in controlling the direction of generational tooth lamina.

A loss of signaling in cylopamine-treated cultures could lead to decreased cell survival and ultimately a shorter dental lamina. Application of Shh antibodies has been shown to increase apoptosis in the neural crest cells (Ahlgren and Bronner-Fraser, 1999). We did see apoptosis in the ectoderm in some cultures, but the most noticeable areas with increased staining were in the mesenchyme. This raises the possibility that the effects of cyclopamine on dental lamina growth are indirect and that the abrogation of Hh signals may have resulted in the loss of a second signal, perhaps from the

Table 1
Effects of cyclopamine treatment on *in vitro* development of *Python regius* dental tissues

Treatment	Total # of anlagen counted (*n*)	Number of tooth anlagen demonstrating phenotypes (%)				
		Inappropriate overall orientation	Disoriented replacement rudiment	No replacement rudiment	Thinner inner enamel epithelium	Cystic stellate reticulum
10 μM cyclopamine	27	21/27 (78)	5/18 (28)	7/26 (27)	12/22 (55)	21/27 (78)
DMSO	30	0	0	3/24 (13)	0	0

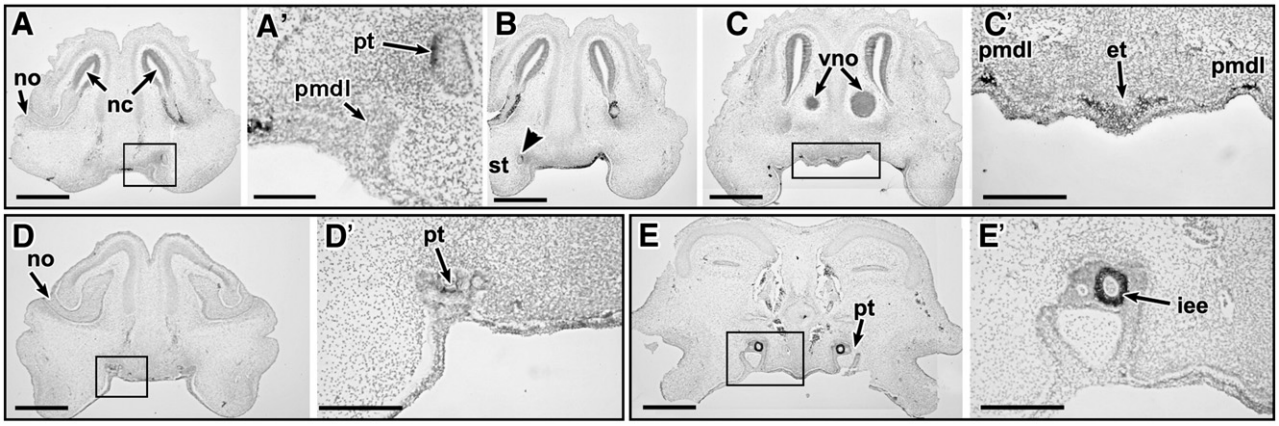

Fig. 9. Expression of *Shh* in premaxillary teeth of *Python regius*. Transverse sections through the premaxilla hybridized with digoxygenin labelled probe. (A–C) Progressively more caudal sections from the same stage 3 embryo showing expression in the midline of the oral cavity and the external part of the nasal cavity but no expression in the premaxillary dental lamina. A bud-stage premaxillary tooth has expression in the inner enamel epithelium (A′). (B) Expression in the premaxillary tooth bud (arrowhead) and stomodeal ectoderm. In panel C, the nascent egg tooth is flanked by premaxillary dental laminae. (D,D′) Stage 4 embryo with two premaxillary teeth at cap stage. *Shh* is beginning in the inner enamel epithelium, but there is no expression in the adjacent oral ectoderm. (E,E′) Stage 6 embryo with more advanced premaxillary teeth with strong expression in the inner enamel epithelium but no expression in the dental lamina or oral ectoderm. Key: et — egg tooth, iee — inner enamel epithelium, nc — nasal cavity, no — nasal orifice, pmdl — premaxillary dentary dental lamina, pt — premaxillary tooth germ, st — stomodeal epithelium, vno — vomeronasal organ. Scale bar for A,B,D,E=500 μm, 200 μm for panels A′,C′D′ and E.

mesenchyme, that promotes cell proliferation or cell survival in the dental lamina epithelium (Fig. 10D). Further gene expression studies will help to identify such candidate signals.

The correlation between oral epithelial *Shh* expression and the depth of tooth-budding from the dental lamina is further corroborated by our *Shh* expression data in the premaxillary teeth of *P. regius*. In these shallow-forming teeth, we could not see persistent *Shh* transcripts at the base of the dental lamina, an expression domain that characterizes deeper-forming teeth at more caudal levels along the jaw. Thus, it is likely that Shh induction of shallow-forming teeth consists of a transitory burst of expression in the odontogenic band that is followed by down-regulation in the oral ectoderm once teeth begin to invaginate. In this regard, expression of *Shh* in the premaxillary teeth of pythons appears to closely resemble that in mammalian teeth (Miyado et al., 2007).

In addition to its role in promoting dental epithelial ingrowth, we propose that the Hh signal from the oral ectoderm controls the angle of the dental lamina relative to the oral surface (Fig. 10B). This hypothesis is based on the straighter dental laminae observed in our cyclopamine-treated cultures. The straightening may well be due to effects on cell proliferation. As we have seen, blocking Hh signaling leads to a more even proliferation pattern within the acute and obtuse sides of the dental lamina (Fig. 10C). It is also possible that apical–basal cell polarity was disrupted in the cyclopamine-treated dental laminae. We saw poorly aligned epithelial cells that appeared to be less polarized than in DMSO controls. Such changes in epithelial cell morphology could have affected dental lamina angle or length.

Shh is required for snake crown morphogenesis

Our organ culture data reveal a further role for *Shh* in the developing snake dentition: patterning and growth of the enamel organ. Successional tooth germs formed in the presence of cyclopamine lack a clearly identifiable inner enamel epithelium and stellate reticulum (Fig. 8E′). *Shh*'s function in the development of the enamel organ also extends beyond its initial establishment. Primary generation teeth formed prior to the start of the culture period display defects in overall size as well as noticeable thinning of the inner enamel epithelium. In our python organ cultures, we could see still see an enamel space and dentin in cyclopamine treated teeth, however some of this matrix was present in the first generation teeth in at the start of our experiment. In the second generation teeth where inner enamel epithelium was just beginning to express *Shh*, cyclopamine treatment inhibited formation of the inner enamel epithelium. Our phenotypes are more severe than those described for the conditional epithelial knockout of *Shh* (Dassule et al., 2000). When mutant teeth were cultured long enough to permit matrix formation in host mice, amelogenesis occurred normally. *Shh* then plays conserved roles in growth and patterning of the enamel organ but the python appears to be more reliant on Hh signalling for ameloblast differentiation than the mouse (Fig. 10D).

The enamel knot is an important signaling centre in mammalian tooth and is critical for elaborating molar cusps. Given these essential roles, it seems likely that an enamel knot or at least a similarly functioning evolutionary homologue would be found in all amniotes. Our studies on the snake show that the tissue from which the enamel knot is derived, the inner enamel epithelium, displays only some of the characteristics of the mammalian enamel knot (*viz*. *Shh* expression, low proliferation). Our observation of *Shh* transcripts and cell apoptosis in the neighboring stellate reticulum raises the possibility that enamel knot function encompasses other tissues in the enamel organ and is not just restricted to the inner enamel epithelium as in mammals. To further validate this hypothesis, a range of genes expressed in the mammalian enamel knot should be cloned and expression scrutinized in developing snake teeth. Our prediction is that other enamel knot genes (e.g., *BMP4*, *LEF-1*) will be expressed throughout the entire inner enamel epithelium as well as in the stellate reticulum.

We acknowledge that the snake tooth is unlikely to be representative of the generalized reptile condition in terms of the enamel knot. Thus, it will also be necessary to extend gene expression and cell dynamic studies to other squamates, particularly those lizard species that have elaborated on the unicuspid morphology (e.g., the gecko *Pavoedura*). Among other lizards, including the skink *Chalcides viridanus* (Delgado et al., 2005) and bearded dragon *Pogona vitticeps* (G. Handrigan, personal obs.), the enamel knot is not a histologically recognizable structure. Furthermore, even though it was reported that an enamel knot was seen in alligators (Westergaard and Ferguson, 1986, 1987), a careful examination of the figures in these reports finds the structure to be poorly defined. Clearly, there is a need for additional studies to determine whether the enamel knot has been retained in reptiles and whether its function has diverged relative to the mammalian knot.

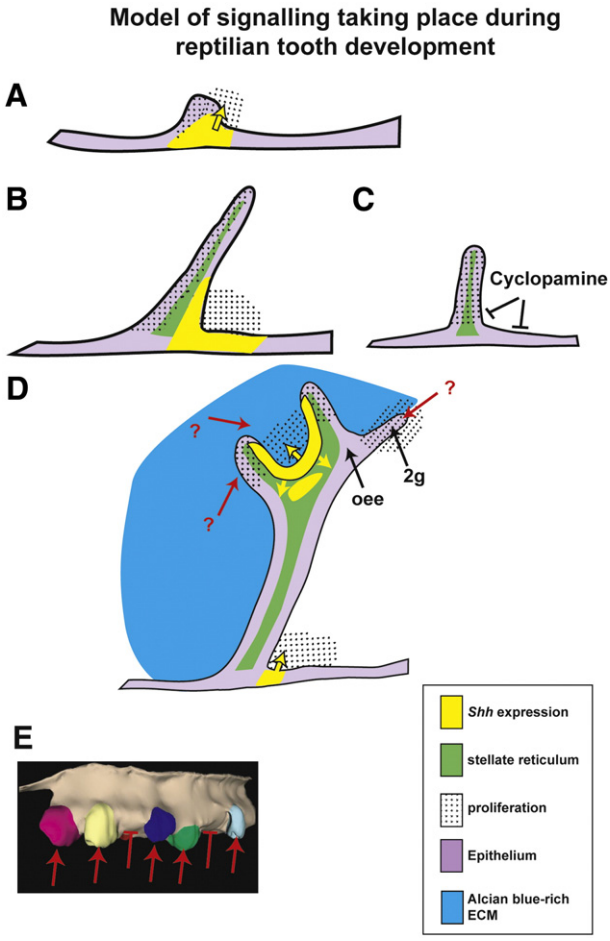

Fig. 10. Model of signaling during snake tooth development. (A) Hh signaling is required for positioning and initiation of the dental lamina as shown with cyclopamine treatment. Asymmetry of proliferation and expression is already present in the early dental lamina. (B) Hh represses proliferation in the acutely angled side of epithelium leading to asymmetry and bending of the dental lamina. The middle layer of cells is likely to form the stellate reticulum. (C) Effect of cyclopamine is to block Hh signaling and to make proliferation even on both sides of the dental lamina. There is also a relative shortening of the dental lamina although proliferation is not decreased overall. (D) Tooth formation occurs and now *Shh* is expressed in the inner enamel epithelium and stellate reticulum. Highest proliferation is in the cervical loops, tip of the generational dental lamina and in the dental papilla. The generational dental lamina 2g originates from the outer enamel epithelium does not express *Shh*. We propose that signals (red question mark) from the mesenchyme, promote outgrowth of the generational dental lamina and formation of teeth on the obtusely angled side. (E) Either the same or different signals from mesenchyme (red arrows) specify tooth formation at certain locations along the dental lamina. It is also equally possible that repressive signals act on the interdental regions where no teeth are formed (red bars). We base the mesenchymal origin for the signal on the fact that interdental epithelia display identical proliferation and gene expression patterns to those areas where teeth form. The lack of differences along the rostro-caudal length of the dental lamina, does not support an ectodermal origin for tooth inducing signals.

Shh is not required to initiate replacement teeth

One of the more striking results of this study was that in the maxillary, palatine and dentary regions teeth form all along the dental lamina and therefore all are connected to each other. Yet it is possible to recognize interdental regions between tooth families in the snake, suggesting that there must be some tissue interaction that determines where the teeth are positioned along the rostral–caudal axis (Fig. 10E). We have excluded *Shh* from the process of specifying tooth locations primarily due to the lack of differential expression along the rostro-caudal length of the dental lamina prior to tooth formation. Furthermore, there is a temporal separation between the invagination of the dental lamina and the timing of formation of the first generation teeth. By the time teeth begin to form, *Shh* expression in the dental lamina has largely receded, remaining only in the junction between oral and dental ectoderm. There is no expression at the tip of the dental lamina, where successional teeth ultimately form (Fig. 10D). *Shh* begins to be expressed later, once replacement teeth can be recognized and then only within the inner enamel epithelium and stellate reticulum. Thus the timing is also not conducive for *Shh* to act as an inducer of succedaneous teeth. It is interesting that a similar conclusion was also reached in regards to the role of Shh in generating successional teeth in fish (Fraser et al., 2006a). In these studies they found that a specific region of the outer dental epithelium gave rise to generational teeth, but this group of cells did not express *Shh*.

There is some debate about the timing of appearance and the origin of the cells that make generational teeth. In the zebrafish, stem cells for making the second generation teeth only appear after the first generation tooth has erupted and originate from the dental crypt epithelium (Huysseune, 2006). However, in certain osteichthyans and amphibians (Davit-Beal et al., 2007; Fraser et al., 2006b) many generations have formed even before the first tooth has erupted into the oral cavity and it is the outer dental epithelium that gives rise to the next generation of teeth. Our data show that snakes also have an outer enamel epithelial origin for the generational dental lamina, as has been reported in humans (Ooë, 1981). Therefore, when searching for the tissues that contain signals to induce the next tooth generation, one should focus on the genes expressed on the obtuse side of the dental lamina, the side that contributes to the outer enamel epithelium. This location for replacement tooth induction signals is supported by the differential staining of the extracellular matrix (Fig. 10D).

In conclusion, our studies of snakes have shown that some aspects of amniote dental patterning are very old in evolutionary terms such as the presence of a primary odontogenic band that expresses *Shh*, the concept that different genes are involved in dental lamina establishment versus tooth induction, and that the outer enamel epithelium is the source of the next generation of teeth. The snake and other reptiles with polyphyodont dentitions present an opportunity to find the elusive signals and genes involved in tooth replacement. In addition, there are many instances where the genetic causes for excess or fewer teeth in humans are not known. What are the signals that induce successional teeth and from which tissue do they originate? Wnts are some of the first candidates that should be examined in future studies (Jarvinen et al., 2006).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.03.004.

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